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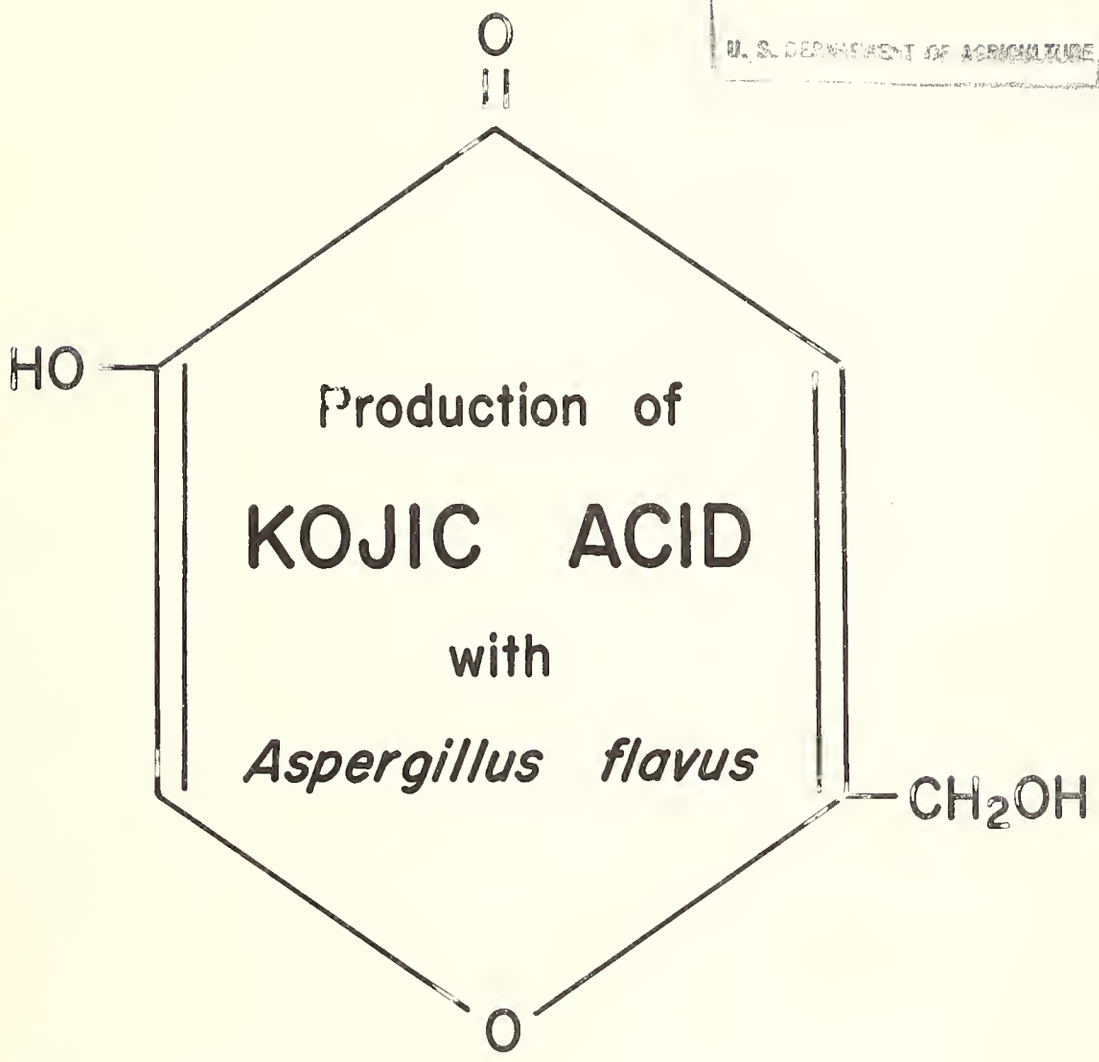

Production of KOJIC ACID with *Aspergillus flavus*

Agricultural Research Service
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The work on the production of kojic acid with Aspergillus flavus was conducted by Dr. Andrew J. Moyer, with the technical assistance of Mrs. Mabel L. Smith and Miss Sinah E. Kelley. Dr. Moyer died February 17, 1959. The material for this bulletin has been prepared by Dr. Robert A. Rhodes and Dr. Richard W. Jackson of the Fermentation Laboratory of the Northern Utilization Research and Development Division, Peoria, Illinois.

PRODUCTION OF KOJIC ACID WITH *ASPERGILLUS FLAVUS*

Kojic acid was first identified by Saito (1)[1/] as a fermentation product of Aspergillus oryzae. Since that time, several investigators have shown that kojic acid can be produced from a wide variety of carbon sources by various molds of the Aspergillus flavus-oryzae [see bibliographies prepared by Barham and Smits (1,2), Prescott and Dunn (15), Foster (7), and Lockwood (12)]. Recent work by Camposano, Chain, and Gualandi (6) has demonstrated that kojic acid can be produced with A. flavus in submerged fermentations but that yields were at least 25 percent less than those obtained in surface culture. Kojic acid has been of interest to the chemist in the synthesis of related organic compounds [see review on formation and properties prepared by Beelik (3)]. Also, some attention has been given to the antibiotic activity of kojic acid and of other metabolic products, such as aspergillic acid produced by this group of molds [Bush and Goth, (5); Jones et al., (9); Waksman and Bugie, (18)].

The production of kojic acid from commercial glucose by a strain of Aspergillus flavus was described by May et al. (13). Later, May et al. (14) found that both kojic acid production and mycelial growth was stimulated by the addition of a small amount of ethylene chlorohydrin to the medium. Barham and Smits (2) reported an improved nutrient medium for the production of kojic acid from xylose.

The kind and concentration of carbon sources, inorganic nutrients, and mold strains used by various investigators differ markedly so that few generalizations can be made as to the best conditions for the production of kojic acid. This bulletin reports an investigation to determine optimal conditions for spore formation and for acid production by A. flavus in surface cultures.

MATERIALS AND METHODS

All experiments reported, unless otherwise stated, were conducted with the strain of A. flavus employed by May et al. (13, 14). This strain is available as NRRL 484 from the ARS Culture Collection of the Northern Utilization Research and Development Division, Peoria, Illinois.

[1/] Underscored numbers in parentheses refer to Literature Cited, p. 10.

Fermentations were conducted at 30° C. in either 200-ml. Erlenmeyer flasks or in 3-liter Fernbach flasks. A commercial grade of glucose monohydrate was employed as the carbon source. When used, ethylene chlorohydrin was added to cool, sterile, culture media just before inoculation. All inoculations were made with dry, ungerminated spores. The methods for the determination of kojic acid and residual glucose were those employed by May et al. (13,14). In the tables and text, glucose content and yield data have been calculated on the basis of anhydrous glucose.

Direct titration values obtained are given in the tabular data inasmuch as significant amounts of acids other than kojic were not detected. Yields of kojic acid, also presented in the tables, were calculated from the titration values and the amount of glucose consumed during the fermentation:

$$\frac{\text{ml. 1 N acid X 142}}{\text{mg. glucose consumed}} \text{ X 100 = \% yield of kojic acid}$$

Since milliliters of 1 N acid formed X 142 equals the weight in milligrams of kojic acid formed, the yield data represent the percentage conversion by weight of glucose to kojic acid.

If it is assumed that 1 mole of glucose yields 1 mole of kojic acid, 0.789 g. of kojic acid theoretically could be formed from each gram of glucose consumed. Therefore, yields based on weight basis may be divided by 0.789 to determine the percent of the theoretical yield obtained.

The mycelium in kojic acid fermentations is especially susceptible to "wetting," i.e., partial or whole submersion; this action results in retarded kojic acid production. Although observations on wetting were recorded routinely, the extent of wetting is mentioned here only when considered to be especially significant.

RESULTS

Spore Production Cultures

Cultures bearing a heavy crop of spores were desirable for rapid and satisfactory inoculations. Consequently, numerous experiments were made to determine the effect of the kind and concentration of nutrients, pH of the medium, and incubation temperature on sporulation, especially in a liquid culture medium. Desired characteristics of a sporulation culture include not only a heavy crop of spores, but also a thin and fragile mycelium which facilitates transfer of the spores for inoculation. Heavy sporulation should

develop in at least 6 days. Two types of sporulation cultures were employed: One grown on an agar medium in test tubes or Petri dishes used for temporary storage of stock cultures, and the other on a liquid medium in Erlenmeyer flasks used to obtain large quantities of spores for inoculating production cultures.

One of the best sporulation media has the following compositional amounts per 1 liter: Commercial glucose, 150.0 g.; "Bacto" peptone, 1.0 g.; KNO_3, 0.5 g.; KH_2PO_4, 0.12 g.; $MgSO_4 \cdot 7H_2O$, 0.1 g.; $ZnSO_4 \cdot 7H_2O$, 0.011 g.; $FeCl_3 \cdot 6H_2O$, 0.1 g.; tap water, 100 ml.; $CaCO_3$, 2.0 g.; and distilled water to make 1 liter. For small cultures, 40-ml. portions were used in 200-ml. Erlenmeyer flasks or 100 ml. in 1-liter flasks. When this medium is gelled with agar, it is satisfactory for use in either test tube slants or Petri dish plates. Sporulation cultures were incubated at 25° to 27° C.

Kojic Acid Production Cultures

Nitrogen Sources and pH Control

The kind and concentration of nitrogen source have a pronounced effect on kojic acid production. As shown in table 1, various single nitrogen sources were supplied to provide 0.35 g. of nitrogen per liter of medium. The nitrogen-containing salts largely determine the pH of the medium during fermentation. Most rapid kojic acid production and greatest fermentation efficiency were obtained with ammonium nitrate. Unequal utilization by the mold of the component ions of ammonium sulfate, ammonium chloride, or ammonium phosphate, brought about a high degree of acidity in the medium which was unfavorable both to growth and to kojic acid production. Sodium or potassium nitrates resulted in a higher pH of the medium, more mycelial growth, and slower fermentation than did ammonium nitrate. Ammonium phosphate was not a satisfactory nitrogen source also because the amounts required to supply sufficient nitrogen to the medium provided an excessive level of phosphate which caused wetting and submerging of the mycelium.

The effect of various concentrations of ammonium nitrate and of sodium phosphate was investigated. Five levels of ammonium nitrate which provided 0.21, 0.28, 0.35, 0.42, and 0.49 g. of nitrogen per liter, were employed in combination with five levels of sodium phosphate, which supplied 21.5, 28.5, 43, 50, and 57 p.p.m. phosphorus, making a total of 25 combinations. A portion of the data obtained from this work is shown in table 2. The amount of acid produced was decreased significantly when less than 0.28 g. nitrogen and 28.5 p.p.m. phosphorus were employed. The optimal levels appear to be 0.35 g. nitrogen per liter and

43 p.p.m. phosphorus. However, within fairly wide ranges of nitrogen and phosphorus concentrations, good yields of kojic acid can be obtained. Wetting and submerging of the mycelium with decreased acid production were encountered with the lower levels of nitrogen and with the highest level of phosphorus.

Some control of the pH of the medium during the fermentation can be attained by using various mixtures of different nitrogen sources. In the experiment presented in table 3, the level of ammonium chloride was kept constant at 0.268 g. per liter, but the ratio of ammonium nitrate to either sodium nitrate or calcium nitrate was varied. The total nitrogen level was 0.280 g. per liter and the initial pH was 4.0. During the fermentation the pH of the medium changed, rising with the higher levels of sodium or calcium nitrates, and falling with an increase in the level of ammonium nitrate. Under these conditions and with ethylene chlorohydrin as an adjunct, the final pH of the medium within the range of 3.6 to 5.1 seemed to have little effect on the amount of acid produced. Poorer acid yields (table 1) at final pH values of 2.0 to 2.1 were obtained with either ammonium chloride or ammonium sulfate as the sole nitrogen sources.

The effect of varying the total nitrogen level with a different mixture of three nitrogen salts used for pH control is shown in table 4. Wetting of the mycelium was encountered at 0.21 g. total nitrogen or less per liter. The phosphorus level of 28.5 p.p.m. used was somewhat below the optimum indicated in tables 2 and 5, and it is possible that higher acid titration values would have been obtained with 40 p.p.m. of phosphorus. At 28.5 p.p.m. of phosphorus, there was no gain in acid production by using more than 0.245 g. per liter of total nitrogen. The final pH of the medium was 3.3 to 3.4. The acid yields obtained are considered to compare favorably with those obtained at higher final pH values as shown in tables 2, 5, and 6.

By and large, better yields of kojic acid are obtained when nitrogen is provided as mixed nitrogen salts to give pH levels of 3.0 to 4.0 than when nitrogen is supplied as ammonium nitrate alone.

Phosphorus Requirements

Table 2 shows that 43 p.p.m. of phosphorus were adequate for fermentation efficiency and high kojic acid yields when favorable nitrogen levels were supplied as ammonium nitrate. Data in table 5 also show the effect of various phosphorus levels when a mixture of nitrogen salts was used with and without ethylene chlorohydrin. In the absence of ethylene chlorohydrin, greatest fermentation efficiency and highest yields were obtained by supplying

57 p.p.m. of phosphorus. When ethylene chlorohydrin was added, the best fermentation occurred at about 34 p.p.m. Wetting of the mycelium was frequently encountered at phosphorus levels above 45 p.p.m.

The data presented in tables 2 and 5 obtained from 9-day fermentations do not show clearly the rate of fermentation at various levels of phosphorus. The acid yields per culture harvested at 2, 4, 6, 8, and 10 days with two levels of phosphorus are shown in table 6. At 45 p.p.m. of phosphorus the fermentation was nearly completed in 8 days with little increase in acid production from 8 to 10 days. A slower but uniform rate of acid production was obtained with 22.5 p.p.m. of phosphorus with as much acid being produced between the 8th and 10th days as was produced between the 6th and 8th days. There was little difference in fermentation efficiency as shown by percentage yields based on glucose consumed. According to the results given in tables 2, 5, and 6, the best phosphorus level appears to be 35-45 p.p.m. with adequate levels of other nutrients and in the presence of ethylene chlorohydrin. There does appear to be some value in using more than 45 p.p.m. of phosphorus if ethylene chlorohydrin is omitted from the medium (see table 5).

Other Nutrient Requirements

Potassium can be supplied by various salts, such as the chloride, nitrate, phosphate, or sulfate. The latter three salts also furnish other nutrients, i.e., nitrogen, phosphorus, and sulfur; consequently, it is difficult to distinguish clearly between the individual effects of these several elements. Potassium chloride was therefore employed as a source of potassium. Mycelial growth increased as potassium was increased. Best conditions for kojic acid production were obtained by supplying 40 to 50 p.p.m. of potassium.

Investigators have usually supplied sulfur and magnesium as $MgSO_4 \cdot 7H_2O$ at either 0.5 g. per liter (2,4,11,13,14) or 0.1 g. per liter (5,10,16). To distinguish more clearly between the requirements for the two elements, sodium sulfate and magnesium chloride were employed as sources of sulfur and magnesium, respectively. Low levels of both magnesium and sulfate caused the mycelium to become wet and to submerge. Sulfur at 20 p.p.m. appears to be adequate for acid production without wetting of the mycelium. No significant increase in mycelial growth or efficiency of the fermentation was obtained by adding more than 3 p.p.m. of magnesium; wetting of the mycelium occurred when only 1 p.p.m. of magnesium was employed.

Iron was found to be essential to good kojic acid yields and was supplied as ferric chloride or ferric tartrate. Little change in mycelial growth or efficiency of the fermentation was brought about by increasing the iron level about 4 p.p.m.

Reference to zinc requirements for kojic acid production by Aspergillus has been made only by Barham and Smits (2), who stated that zinc increased kojic acid production. May et al. (13,14) obtained good yields from commercial glucose without including either iron or zinc in the medium. It seems probable that the grade of commercial glucose used in the present investigation contained less iron and zinc than did the glucose used by May and coworkers. During our investigations the stimulating action of zinc in the fermentation of commercial glucose was demonstrated many times, but the amount of stimulation due to zinc varied considerably. Adequate zinc for good mycelial growth apparently was present in the production medium as an impurity; there was little advantage in adding more than 5 p.p.m. of zinc to stimulate acid production. The effect of zinc and ethylene chlorohydrin on kojic acid production is shown in table 7. The stimulating action of zinc was greater than that obtained with ethylene chlorohydrin; best acid production resulted when both zinc and ethylene chlorohydrin were present.

Successful kojic acid fermentations were obtained at glucose levels of 10 to 33 percent, but most rapid and efficient fermentations occurred when the initial glucose level was between 20 to 24 percent. At initial glucose levels of less than 10 percent there was a tendency for increased mycelial growth and reduced efficiency in kojic acid production.

Addition of corn steep liquor to the medium did not improve kojic acid yields by strain NRRL 484.

Kojic Acid Production in Large Flasks

A large number of cultures were grown in 3-liter Fernbach flasks each containing 750 ml. of the culture medium. The nutrient requirements closely paralleled those found best in the small flasks; however, it was possible to use slightly lower nutrient levels as the depth of the medium was increased. Fifty pounds of kojic acid were recovered from these fermentations. The formation of large crystals of kojic acid under the mycelium is shown in figure 1. The medium employed in this flask is considered to be optimum and had the following composition per 1 liter: Commercial glucose monohydrate, 260.0 g.; NH_4NO_3, 0.8 g.; NH_4Cl, 0.134 g.; $NaH_2PO_4 \cdot H_2O$, 0.191 g.; $MgCl_2 \cdot 6H_2O$, 0.05 g.; KCl, 0.15 g.; Na_2SO_4, 0.1 g.; $ZnSO_4 \cdot 7H_2O$, 0.022 g.; ferric tartrate, 0.02 g.; ethylene chlorohydrin, 0.1 ml.; and potato starch,

0.5 g. The flask shown in the figure contained initially 750 ml. of medium (169 g. anhydrous glucose) and was incubated at 30° C. for 14 days. Data at harvest were: 166.0 g. glucose consumed; 81.3 g. of kojic acid produced; weight yield of kojic acid calculated on glucose consumed was 49 percent or a theoretical yield of 62 percent based on glucose consumed; the dry weight of mycelium was 4.2 g., and final pH of the medium, 3.5.

Kojic Acid Production by Other Strains

During the past 25 years, kojic acid production by approximately 50 strains of the A. flavus-oryzae group has been studied, and strain NRRL 484 was the most efficient organism found. Some degenerate forms of this strain were isolated during these investigations; they usually sporulated poorly and in fermentations gave heavy mycelial growth and low yields of acid.

Another effective strain, NRRL 1949, produced 46 g. of kojic acid from 198 g. of glucose in 8 days on a medium similar to that discussed in connection with figure 1, except that 2 ml. of corn steep liquor were added. This strain is reported to produce aspergillic acid (9), but no data were obtained on the amount produced under these conditions.

DISCUSSION

The advantages of using mold cultures that form large numbers of spores are obvious when large-scale inoculation of surface cultures is necessary. The entire surface of the medium must be well seeded, since the radial growth of the mold is too slow to spread over any sizable unseeded area. The nutrients necessary for abundant sporulation are approximately the same as those optimal for kojic acid production. The sporulation medium contains a lower nitrogen level and has an initially higher pH value. Good sporulation required a lower temperature than was found to be best for kojic acid production.

Barham and Smits (2) and Katagiri (10) believed that the optimum pH of the medium was about 2.5, whereas Tamiya (17) obtained maximum acid production at pH 5.5. These opposing conclusions may result either from differences in mold strains or from an inadequate distinction between the initial pH and the pH developed during the fermentation. In our experience changes in the pH of the medium during the course of the fermentation were primarily associated with the type of nitrogen source employed. An optimum pH has not been established, but our studies show that good kojic acid production can be obtained under conditions such

that the final pH of the medium varies from 3.3 to 5.1. A proper mixture of suitable nitrogen sources can be employed to maintain the pH of the medium within this range.

Suboptimal levels of magnesium, sulfur, and nitrogen resulted in wetting and submerging of the mycelium. However, the wetting and submerging of the mycelium with concurrent decrease in acid production also are associated with other than nutritional factors. The work of Camposana et al. (6) has shown that the production of kojic acid by A. flavus in submerged culture was strongly influenced by mechanical agitation under aeration conditions which gave near saturation levels of oxygen in the medium. Mechanical agitation had a pronounced effect on the morphological structure of mold mycelium. This effect could be correlated with effective kojic acid production; no kojic acid formation took place under agitation conditions sufficient to maintain the mycelium in a state of branching hypha and heavy cell wall development. In surface cultures also the morphological structure of the mycelial mat is influenced by conditions of culture and the formation of kojic acid thereby affected. For example, the mycelium produced from inoculation of fermentation flasks with few spores, as contrasted with many, usually did not become wet. Light inoculation resulted in a slightly slower mycelial growth for the first 2 days. The addition of 0.5 to 2.0 g. of potato starch per liter of production medium was very effective in preventing mycelium wetting, especially when higher levels of phosphorus were employed.

It should be recognized that the maintenance of mycelial growth on the surface of liquid media probably depends both on the nutritional state of the mold and on certain physical properties of the medium, such as osmotic pressure and surface tension. The amount of any salt present in the medium may influence wetting of the mycelium by influencing either of these aspects. Thus, the mold may require a finite amount of phosphorus for vigorous acid production, a larger amount for vigorous growth, and a still different amount for the maintenance of a cell surface that prevents wetting of the mycelium. Related to the latter, of course, is the effect which phosphorus compounds or their radicals may have on the physical state of the medium at these concentrations. Differences in the availability of phosphorus in its various salts also influence the amount of phosphorus required in the medium. Probably optimal mineral requirements are only compromises between these and other interrelated phenomena.

The nature of the ethylene chlorohydrin stimulation of kojic acid production is not known. Foster (7) suggested that this chemical might enhance cell permeability. The increase in amount of mycelial growth in the presence of ethylene chlorohydrin reported by May et al. (14) was not confirmed consistently during

these investigations. In most fermentations the addition of ethylene chlorohydrin caused a decided inhibition of both mycelial growth and spore formation. The action of ethylene chlorohydrin apparently was dependent on the amount and manner of supply of nutrients. Stimulation of kojic acid production was most pronounced in cultures having either sodium nitrate or calcium nitrate as the main sources of nitrogen, where heavy sporulation occurred in the absence of ethylene chlorohydrin. The stimulating action became less as the supply of phosphorus was increased. The stimulating action of zinc in some culture media was as great as that obtained with ethylene chlorohydrin; fastest fermentations were obtained when both of these compounds were present in the medium. Yields of over 51 percent of theory, based on glucose consumed, were obtained with proper nutrient conditions in the presence of ethylene chlorohydrin.

The production of kojic acid by strain NRRL 1949 is of interest since Jones et al. (9) reported no significant yield of kojic acid by this strain in their investigations on aspergillic acid. Corn steep liquor was more effective in promoting kojic acid production by strain NRRL 1949 than by strain NRRL 484. The value of employing such nutrients may depend upon the mold strain employed. In this connection, reference is made to the report of Jennings and Williams (8) who obtained good yields of kojic acid from Aspergillus effusus (of the flavus-oryzae group) in a medium containing maltose, peptone, and malt extract.

The time required for complete fermentation depends upon both proper nutrients and depth of the culture medium. It would be highly desirable to decrease substantially the fermentation time of 2 weeks required with 750 ml. of medium containing 20 to 25 percent glucose in the large Fernbach flasks.

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SUMMARY

Improved procedures have been developed for the production of the spores required for inoculation of cultures used to produce kojic acid.

Nutritional factors affecting the production of kojic acid by surface cultures of Aspergillus flavus NRRL 484 were investigated. Optimal levels of phosphorus, potassium, sulfur, magnesium, nitrogen, iron, and zinc were established. Ammonium nitrate was the best single source of nitrogen; however, mixtures of nitrogen sources employed to influence the pH of the medium were equally satisfactory. Both zinc and ethylene chlorohydrin stimulated production of kojic acid without a corresponding stimulation of mycelial growth. In large flask cultures the weight yield of kojic acid, calculated on glucose consumed, was 48.9 percent, corresponding to 62.1 percent of theoretical.

Wetting and submerging of the mycelium with concurrent decrease in acid production could be greatly diminished by use in the medium of suitable nutrients, a small amount of potato starch, and a light spore inoculum.

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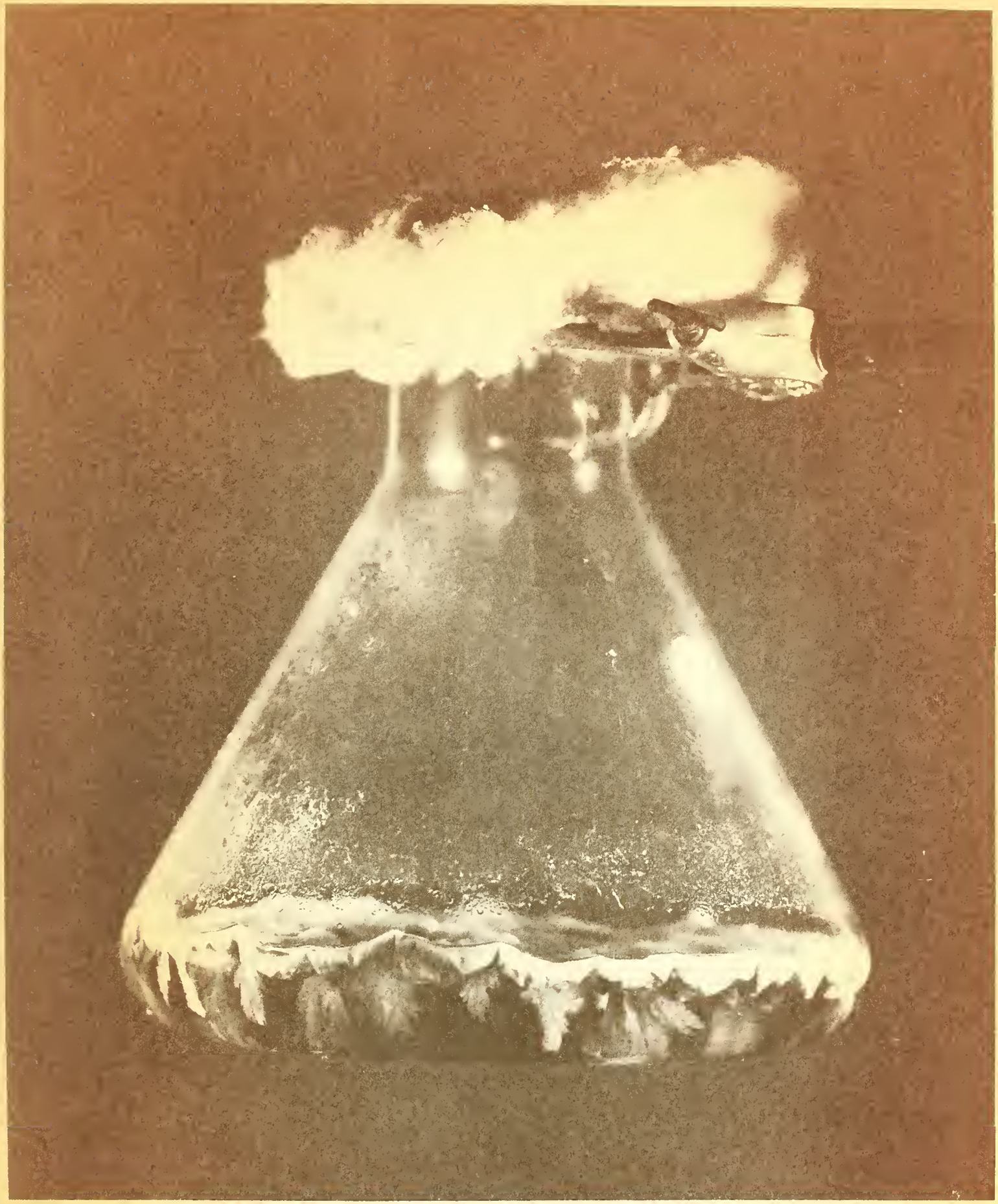

Figure 1. Formation of kojic acid crystals in the medium under the mycelium of *Aspergillus flavus* NRRL 484.

TABLE 1.--Effect of nitrogen source on kojic acid production

Nitrogen source	Mycelial weight per culture	pH		Glucose consumed per culture	0.1N acid formed per culture	Kojic acid yield[1/]
		Initial	Final			
	g.			g.	ml.	%
NH_4NO_3	0.26	4.2	4.3	9.05	312	49
$(NH_4)_2HPO_4$	.20	6.3	2.7	4.19	120	41
$(NH_4)_2SO_4$	.19	4.4	2.1	3.27	100	43
NH_4Cl	.19	4.3	2.0	3.43	122	43
$NaNO_3$	.28	4.2	5.3	6.98	234	47
KNO_3	.28	4.2	5.2	8.30	252	43

[1/] As stated in the text and as used in tabular data, kojic acid yields were calculated as follows:

$$\frac{\text{ml. 1N acid} \times 142}{\text{mg. glucose consumed}} \times 100 = \% \text{ yield of kojic acid based on glucose consumed}$$

Culture medium, amounts per liter: Glucose, 194.0 g. (9.7 g. per culture); nitrogen, 0.35 g. as salts indicated; KH_2PO_4, 0.15 g.; $MgSO_4 \cdot 7H_2O$, 0.125 g.; $ZnSO_4 \cdot 7H_2O$, 0.044 g.; iron tartrate, 0.02 g.; ethylene chlorohydrin, 0.15 ml.

Culture volume: 50 ml. in 200-ml. Erlenmeyer flask.

Age at harvest: 8 days.

TABLE 2.--Effect of concentration of NH_4NO_3 and NaH_2PO_4 on kojic acid production

Nitrogen supplied	Phosphorus supplied	Mycelial weight per culture	pH (Final)	Glucose consumed per culture	0.1N acid formed per culture	Kojic acid yield 1/
g./l.	p.p.m.	g.		g.	ml.	%
0.28	28.5	0.27	4.5	10.47	353	48
	43	.42	4.5	--	377	--
	50	.41	4.5	12.86	413	46
.35	28.5	.35	4.6	11.70	389	47
	43	.39	4.5	13.23	444	48
	50	.41	4.5	12.77	398	44
.49	28.5	.39	4.5	--	384	--
	43	.44	4.5	13.10	401	43
	50	.42	4.6	13.07	425	44

1/ Culture medium, amounts per liter: Glucose, 231.0 g. (13.86 g. per culture); $MgCl_2 \cdot 6H_2O$, 0.05 g.; K_2SO_4, 0.2 g.; $ZnSO_4 \cdot 7H_2O$, 0.022 g.; iron tartrate, 0.02 g.; ethylene chlorohydrin, 0.15 ml.; potato starch, 1.0 g.

Culture volume: 60 ml. in 200-ml. Erlenmeyer flask.

Age at harvest: 9 days.

TABLE 3.--Effect of combinations of nitrogen sources and ethylene chlorohydrin on kojic acid production

Nitrogen salts supplied			Ethylene chlorohydrin	Mycelial weight per culture	pH (Final)	Glucose consumed per culture	0.1N acid formed per culture	Kojic acid yield[1]
$NaNO_3$	$Ca(NO_3)_2 \cdot 4H_2O$	NH_4NO_3						
g./l.	g./l.	g./l.	ml./l.	g.		g.	ml.	%
0.850	0	0.20	0	0.36	4.9	3.76	98	37
.850	0	.20	0.1	.23	5.1	9.32	342	52
.317	0	.45	0	.29	3.7	8.68	266	43
.317	0	.45	.1	.26	3.6	10.05	357	50
0	1.180	.20	0	.45	4.6	4.33	89	29
0	1.180	.20	.1	.25	4.8	9.82	365	53
0	0.443	.45	0	.38	3.8	7.49	212	40
0	.443	.45	.1	.25	3.6	9.36	333	50

[1] Culture medium, amounts per liter: Glucose, 215.0 g. (12.9 g. per culture); NH_4Cl, 0.268 g. (total N supplied, 0.280 g. per l.); $NaH_2PO_4 \cdot H_2O$, 0.151 g.; $MgCl_2 \cdot 6H_2O$, 0.03 g.; Na_2SO_4, 0.10 g.; KCl, 0.137 g.; $ZnSO_4 \cdot 7H_2O$, 0.044 g.; iron tartrate, 0.02 g.

Culture volume: 60 ml. in 200-ml. Erlenmeyer flask.

Age at harvest: 9 days.

TABLE 4.--Effect of nitrogen level as supplied by a mixture of three nitrogen sources on kojic acid production

Nitrogen sources			Total nitrogen supplied	Mycelial weight per culture	pH (Final)	Glucose consumed per culture	0.1N acid formed per culture	Kojic acid yield[1]
$NaNO_3$	NH_4NO_3	NH_4Cl						
g./l.	g./l.	g./l.	g./l.	g.		g.	ml.	%
0.127	0.220	0.161	0.140	0.18	3.1	4.39	86	27
.159	.275	.201	.175	.24	3.3	7.61	233	43
.191	.330	.241	.210	.28	3.4	10.76	374	50
.223	.385	.281	.245	.31	3.4	11.30	398	50
.255	.440	.322	.280	.34	3.3	11.31	389	49

[1] Culture medium, amounts per liter: Glucose, 216.0 g. (12.96 g. glucose per culture); $NaH_2PO_4 \cdot H_2O$, 0.127 g.; $MgCl_2 \cdot 6H_2O$, 0.02 g.; Na_2SO_4, 0.1 g.; KCl, 0.07 g.; $ZnSO_4 \cdot 7H_2O$, 0.044 g.; iron tartrate, 0.02 g.; ethylene chlorohydrin, 0.1 ml.

Culture volume: 60 ml. in 200-ml. Erlenmeyer flask.

Age at harvest: 9 days.

TABLE 5.--Effect of phosphorus concentration and ethylene chlorohydrin on kojic acid production

Ethylene chlorohydrin	Phosphorus supplied	Mycelial weight per culture	pH (Final)	Glucose consumed per culture	0.1 acid formed per culture	Kojic acid yield[1]
ml./l.	p.p.m.	g.		g.	ml.	%
0	17	0.31	4.0	7.60	237	39
	34	.29	3.9	10.23	315	43
	45	.29	4.0	10.68	345	46
	57	.31	4.0	11.52	378	47
0.15	17	.24	4.0	8.94	329	52
	34	.30	4.0	11.53	408	51
	45	.30	4.1	11.64	412	49
	57	.30	4.1	12.22	416	48

[1] Culture medium, amounts per liter: Glucose, 210.0 g. (12.6 g. per culture); NH_4NO_3, 0.54 g.; $NaNO_3$, 0.39 g.; NH_4Cl, 0.24 g. (total N, 0.33 g.); phosphorus as $NaH_2PO_4 \cdot H_2O$; $MgCl_2 \cdot 6H_2O$, 0.03 g.; Na_2SO_4, 0.10 g.; KCl, 0.137 g.; $ZnSO_4 \cdot 7H_2O$, 0.044 g.; iron tartrate, 0.02 g.

Culture volume: 60 ml. in 200-ml. Erlenmeyer flask.

Age at harvest: 9 days.

TABLE 6.--Effect of levels of phosphorus on the rate of kojic acid production

Phosphorus supplied	Culture age	Mycelial weight per culture	pH (Final)	Glucose consumed per liter	0.1N acid formed per culture	Kojic acid yield[1]
p.p.m.	days	g.		g.	ml.	%
22.5	2	0.13	3.5	2.09	36	24
	4	.21	3.5	4.46	129	38
	6	.25	3.4	6.40	197	43
	8	.28	3.5	8.10	276	48
	10	.31	3.5	9.61	339	50
45.0	2	.17	3.5	2.28	48	29
	4	.29	3.5	6.79	212	44
	6	.35	3.5	10.08	330	47
	8	.38	3.5	12.31	422	48
	10	.35	3.5	12.66	437	49

[1] Culture medium, amounts per liter: Glucose, 224.0 g. (13.44 g. per culture); NH_4NO_3, 0.50 g.; $NaNO_3$, 0.425 g.; NH_4Cl, 0.402 g. (total nitrogen, 0.350 g.); phosphorus as $NaH_2PO_4 \cdot H_2O$; $MgCl_2 \cdot 6H_2O$, 0.03 g.; Na_2SO_4, 0.1 g.; KCl, 0.137 g.; $ZnSO_4 \cdot 7H_2O$, 0.066 g.; iron tartrate, 0.02 g.; ethylene chlorohydrin, 0.15 ml.

Culture volume: 60 ml. in 200-ml. Erlenmeyer flask.

TABLE 7.--Effect of ethylene chlorohydrin and zinc on kojic acid production

Ethylene chlorohydrin	Zinc supplied	Mycelial weight per culture	pH (Final)	Glucose consumed per culture	0.1N acid formed per culture	Kojic acid yield[1/]
ml./l.	p.p.m.	g.		g.	ml.	%
0	0	0.38	4.0	9.67	276	41
0	5	.37	4.0	12.78	379	42
0.1	0	.40	3.9	11.80	355	43
.1	5	.44	4.0	13.57	437	46

1/ Culture medium, amounts per liter: Glucose, 232.0 g. (13.9 g. glucose per culture); NH_4NO_3, 0.9 g.; $NaH_2PO_4 \cdot H_2O$, 0.254 g.; $MgCl_2 \cdot 6H_2O$, 0.05 g.; K_2SO_4, 0.232 g.; iron tartrate, 0.003 g.

Culture volume: 60 ml. in 200-ml. Erlenmeyer flask.

Initial pH: 4.1.

Age at harvest: 10 days.